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PCT/KR2005/000235

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

(Chapter II of the Patent Cooperation Treaty)

(PCT Artcle 36 and Rule 70)

Applicant's or agent's file reference PCT-2758	**FOR FURTHER ACTION**	See Form PCT/IPEA/416
International application No. **PCT/KR2005/000235**	International filing date *(day/month/year)* **27 JANUARY 2005 (27.01.2005)**	Priority date *(day/month/year)* 30 JANUARY 2004 (30.01.2004)

International Patent Classification (IPC) or national classification and IPC

C12N 9/24(2006.01)i, C12N 9/30(2006.01)i, C12N 15/56(2006.01)i, C12N 15/63(2006.01)i, C12N 9/00(2006.01)i

Applicant

LIFENZA CO., LTD. et al

1. This report is the international preliminary examination report, established by this International Preliminary Examining Authority under Article 35 and transmitted to the applicant according to Article 36.
2. This REPORT consists of a total of 4 sheets, including this cover sheet.
3. This report is also accompanied by ANNEXES, comprising:
   a. ☒ (sent to the applicant and to the International Bureau) a total of 7 sheets, as follows:
      ☒ sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications authorized by this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions).
      ☐ sheets which supersede earlier sheets, but which this Authority considers contain an amendment that goes beyond the disclosure in the international application as filed, as indicated in item 4 of Box No. I and the Supplemental Box.
   b. ☐ *(sent to the International Bureau only)* a total of (indicate type and number of electronic carrier(s)) _____, containing a sequence listing and/or tables related thereto, in electronic form only, as indicated in the Supplemental Box relating to Sequence Listing (see Section 802 of the Administrative Instructions).

4. This report contains indications relating to the following items:
   - ☒ Box No. I Basis of the report
   - ☐ Box No. II Priority
   - ☐ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - ☐ Box No. IV Lack of unity of invention
   - ☒ Box No. V Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - ☐ Box No. VI Certain documents cited
   - ☐ Box No. VII Certain defects in the international application
   - ☐ Box No. VIII Certain observations on the international application

Date of submission of the demamd	Date of completion of this report
24 AUGUST 2005 (24.08.2005)	16 MAY 2006 (16.05.2006)

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Box No. I Basis of the report

1. With regard to the **language,** this report is based on the international application in the language in which it was filed, unless otherwise indicated under this item.

☐ This report is based on translations from the original language into the following language _____, which is the language of a translation furnished for the purposes of:

- ☐ international search (under Rules 12.3 and 23.1(b))
- ☐ publication of the international application (under Rule 12.4)
- ☐ international preliminary examination (under Rules 55.2 and/or 55.3)

2. With regard to the **elements** of the international application, this report is based on *(replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this reort as "originally filed" and are not annexed to this report):*

☐ the international application as originally filed/furnished

☒ the description:
pages 1-5, 9-11, 13-22 as originally filed/furnished
pages* 6-8, 12 received by this Authority on 07/04/2006
pages* _____ received by this Authority on _____

☒ the claims:
pages 24 as originally filed/furnished
pages* _____ as amended (together with any statment) under Article 19
pages* 23 received by this Authority on 07/04/2006
pages* _____ received by this Authority on _____

☒ the drawings:
pages 1/7-7/7 as originally filed/furnished
pages* _____ received by this Authority on _____
pages* _____ received by this Authority on _____

☒ the sequence listing and/or any related table(s) - see Supplemental Box Relating to Sequence Listing.

3. ☐ The amendments have resulted in the cancellation of:
- ☐ the description, pages _____
- ☐ the claims, Nos. _____
- ☐ the drawings, sheets _____
- ☐ the sequence listing *(specify)* : _____
- ☐ any table(s) related to sequence listing *(specify)* : _____

4. ☐ This report has been established as if (some of) the amendments annexed to this report and listed below had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).
- ☐ the description, pages _____
- ☐ the claims, Nos. _____
- ☐ the drawings, sheets _____
- ☐ the sequence listing *(specify)*: _____
- ☐ any table(s) related to sequence listing *(specify)* : _____

* *If item 4 applies, some or all of those sheets may be marked "superseded."*

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Box No. V Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	1-10	YES
	Claims	None	NO
Inventive step (IS)	Claims	1-10	YES
	Claims	None	NO
Industrial applicability (IA)	Claims	1-10	YES
	Claims	None	NO

2. Citations and explanations (Rule 70.7)

The following documents have been considered for the purpose of this report:

D1: WO 2003/018790 A1 (LIFENZA CO., LTD.) 6 MARCH 2003
D2: WO 2001/066570 A1 (KIM et al.) 13 SEPTEMBER 2001
D3: J. Microbiol. Biotechnol., Vol. 9(3), pp. 260-264 (1999)
D4: Biosci. Biotechnol. Biochem., Vol. 64(2), pp. 223-228 (2000)

The present invention relates to an enzyme, having the amino acid sequence of SEQ. ID. NO:1, with the activity of hydrolyzing amylopectin, starch, glycogen and amylose; a gene (SEQ. ID. NO:2) encoding said enzyme; a transformed cell expressing said gene; a method of producing said enzyme; and a composition for the dextran removal and the plaque elimination.

D1-D4 disclose the DEXAMmase (dextranase and amylase), having antiplaque and anticaries activities, having dextranase and amylase activities simultaneously and degrading insoluble glucans, from *Lipomyces starkeyi* KSM 22 (KFCC 11077); a preparation method of DEXAMase; and an oral composition comprising the same.

However, none of the prior art documents disclose the amino acid sequence of the enzyme (SEQ. ID. NO:1) capable of hydrolyzing amylopectin, starch, glycogen and amylose, and the nucleotide sequence of gene (SEQ. ID. NO:2) encoding the enzyme, and said enzyme in this invention cannot be derived in an obvious manner from the prior art documents.

Therefore, claims 1-10 meet the requirements of novelty, inventive step and industrial applicability under PCT Article 33(2)-(4). //

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Supplemental Box Relating to Sequence Listing

Continuation of Box No. I, item 2:

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, this report was established on the basis of:

   a. type of material
   - [x] a sequence listing
   - [ ] table(s) related to the sequence listing

   b. format of material
   - [x] on paper
   - [ ] in electronic form

   c. time of filing/furnishing
   - [ ] contained in the international application as filed
   - [ ] filed together with the international application in electronic form
   - [ ] furnished subsequently to this Authority for the purposes of search and/or examination
   - [x] received by this Authority as an amendment* on 07/04/2006

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table(s) relating thereto has been filed of furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

maltodextrin (Mn), before and after being hydrolyzed by the enzyme (lanes 1 and 2 in panel A, respectively) and maltooligosaccharide samples (1% w/v) are analyzed after purified LSA is allowed to react with a series of maltooligosaccharides including G1 (glucose) to G7 (maltoheptaose) (lanes 1 to 7 in panel B, respectively).

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The acquisition of a gene coding for the carbohydrolase (LSA) of the present invention starts by culturing *Lipomyces starkeyi* in a medium containing starch. Next, on the basis of N-terminal amino acid sequences of carbohydrate hydrolyzing enzymes purified from *L. starkeyi*, primers comprising expected conserved regions are constructed, followed by PCR with the primers. The PCR product, approximately 2 kb long, is used for 5′ RACE and 3′ RACE to allow for a complete carbohydrolase gene (LSA). After being amplified by PCR, the gene is cloned in the vector pRSETB (Invitrogen, U.S.A.) with which *Escherichia coli* DH5@/pRLSA is then transformed.

L. starkeyi is known to produce endo-dextranase (EC 3.2.1.11) which degrades dextran and α-amylase which degrades starch. This microorganism has been applied to foods and not yet reported to produce antibiotics or other toxic metabolites.

Most of the dextranases produced by microorganisms, except for a few derived from bacteria, are known as inducible enzymes. *L. starkeyi* ATCC74054, reported first in U.S. Pat.

No. 5,229,277, produces both dextranase and amylase whose characteristics are also disclosed. It is also reported that the strain produces low molecular weight dextrans from sucrose and starch. On the basis of the findings, the present inventors have acquired Korean Pat. No. 10-0358376 on Oct. 11, 2002 (corresponding to U.S. Pat. No. 6,485,953 dated Nov. 26, 2002) which relates to a DXAMase enzyme capable of hydrolyzing both dextran and starch, a microorganism producing the enzyme (identified as *Lipomyces starkeyi* KFCC-11077), and a composition comprising the enzyme.

The enzyme expressed from the gene (*lsa*) of the present invention is a carbohydrolase capable of hydrolyzing amylopectin, starch, glycogen and amylose. Also, the enzyme according to the present invention is found to degrade dextran, alpha-cyclodextrin and pullulan. The enzyme is highly stable. Not only is its activity 90% of its maximum over a relatively broad pH range (pH 5-8), but also it is not inhibited even by a denaturation solution such as an EGTA-containing solution. Ca^{2+} or Mg^{2+} serves as a cofactor for the enzyme.

Also, the present invention is directed to a novel microorganism carrying the gene coding for the carbohydrolase. The strain *Escherichia coli* DH5@/pRLSA according to the present invention was deposited in the Korean Collection for Type Cultures (KCTC) located in Yusung Gu, Daejeon City, South Korea, with the accession number of KCTC 10573BP, on Dec. 24, 2003.

Also, the present invention is directed to a method of

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producing the carbohydrolase. First, the strain *Escherichia coli* DH5@/pRLSA is cultured. After being harvested from the culture, the cells are disrupted using glass beads to isolate the carbohydrolase therefrom.

A composition comprising the enzyme of the present invention may be used in a variety of oral care applications. By virtue of its ability to degrade polysaccharides such as dextran and amylose, the enzyme of the present invention is also effectively used to remove dextran during sugar production. Additionally, compositions comprising the enzyme according to the present invention can be applied to foods such as gum, drinks, milks, etc. and their constituents may be readily determined by those who are skilled in the art.

A better understanding of the present invention may be obtained through the following examples which are set forth to illustrate, but are not to be construed as the limit of the present invention.

EXAMPLE 1: *lsa* gene cloning in *Lipomyces starkeyi*

1) Strain and plasmid

Lipomyces starkeyi KFCC 11077, which produces DXAMase having dextranase and amylase activity, was used as a DNA donor for cDNA isolation and amylase gene selection. General DNA manipulation and DNA sequencing were carried out with *Escherichia coli* DH5α and pGEM-T easy (Promega, USA). For the construction of a cDNA library, *E. coli* XL1-Blue and SOLR (Stratagene, USA) were used as host cells with lambda phase

primer 5'-CTCTACATGGAGCAGATTCCA-3' which respectively correspond to N-terminal and C-terminal amino acid sequences of the protein showing dextranase and amylase characteristics. After being separated on agarose gel, the PCR product was purified with an AccPrep™ gel extraction kit (Bioneer, Korea) and ligated with pGEM-T easy vector (Promega, USA). Base sequencing was performed using ABI PRISM Cycle Sequencing Kit (Perkin Elmer Corp. USA) in a GeneAmP 9600 thermal cycler DNA sequencing system (Model 373-18, Applied Biosystems, USA).

8) Heterologous expression and purification of LSA protein in *E. coli*

The gene *lsa* was inserted into the SacI-EcoRI site of pRSETB vector (Invitrogen USA) to prepare a recombinant vector pRSET-LSA. *Escherichia coli* DH5@/pRLSA transformed with pRSET-LSA was cultured at 37°C to a midstationary phase in an LB medium containing 50 mg/l ampicillin. After the addition of IPTG to the culture to a final concentration of 1 mM, incubation was carried out at 28°C for 6 hours. Cells were harvested by centrifugation (5000 g x 10 min), washed with 0.1 M potassium phosphate (pH 7.4 and lyzed by sonication. Purification of the expressed protein was performed with Ni^{2+}-nitrilotriacetic acid-agarose (NTA) (Quiagene, Germany). The cell lysate was combined with Ni^{2+}-NTA and allowed to stand for 1 hour at 4°C, and the mixture was loaded onto a column which was then washed four times with a washing buffer. Each 0.5 ml of the protein fraction was emulsified with a buffer.

WHAT IS CLAIMED IS:

1. A protein, comprising an amino acid sequence of SEQ. ID. No. 1, which has the activity of hydrolyzing amylopectin, starch, glycogen and amylose, a derivative thereof, or a fragment thereof.

2. A gene of SEQ. ID. No. 2, encoding the protein, the derivative, or the fragment of claim 1, a derivative thereof, or a fragment thereof.

3. A transformed cell, expressing the gene, the derivative, or the fragment of claim 2.

4. The transformed cell as defined in claim 2, wherein the cell is prokaryotic or eukaryotic.

5. The transformed cell as defined in claim 3 or 4, wherein the cell is *Escherichia coli* DH5@/pRLSA deposited with the accession number of KCTC 10573BP.

6. A method of producing an enzyme having activity of hydrolyzing amylopectin, starch, glycogen and amylose, comprising:

culturing the cell of claim 3;

expressing the enzyme in the cultured cell; and

purifying the expressed enzyme.

【Sequence Listing】

<110> Lifenza Co., Ltd.

<120> PROTEIN WITH ACTIVITY OF HYDROLYZING AMYLOPECTIN, STARCH, GLYCOGEN AND AMYLOSE, GENE ENCODING THE SAME, CELL EXPRESSING THE SAME, AND PRODUCTION METHOD THEREOF

<150> KR2004-0006186

<151> 2004-01-30

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<211> 647

<212> PRT

<213> Artificial Sequence

<220>

<223> *Escherichia coli* DH5@/pRLSA

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20 25 30

Val Thr Val Leu Ser Ser Pro Glu Ser Val Thr Ser Ser Asn His Val
35 40 45

Glu Leu Ala Ser His Glu Met Cys Asp Ser Thr Leu Ser Ala Ser Leu
50 55 60

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IPEA/KR 0 7. 0 4. 2006.

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<213> Artificial Sequence

<220>
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tcagcgtccc tttatatcta caatgatgat tatgataaga ttgtgacact ttattatctt 240

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tacatccaag gcatgggctt tactgctatt tggatttctc ctatagtgga aaatattccc 720

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